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Immunohistochemical Detection of Aflatoxin B_1–DNA Adducts and Hepatitis B Virus Antigens in Hepatocellular Carcinoma and Nontumorous Liver Tissue

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Monoclonal antibodies recognizing the stable imidazole ring-opened form of the major N^7-guanine aflatoxin B_1–DNA adduct have been used in competitive enzyme-linked immunosorbent assays (ELISA) and indirect immunofluorescence assays to quantitate adduct levels in liver tissue. Methods were developed in AFB_1-treated animals, then applied to paired tumor and nontumor liver tissues of hepatocellular carcinoma patients from Taiwan. An avidin–biotin complex staining method was also used for of the detection of hepatitis B surface (HBsAg) and X (HBxAg) antigens in liver sections. A total of 8 (30%) hepatocellular carcinoma (HCC) samples and 7 (26%) adjacent nontumor liver tissue samples from Taiwan were positive for AFB_1–DNA adducts. For HBsAg, 10 (37%) HCC samples and 22 (81%) adjacent nontumorous liver samples were positive, and 9 (33%) HCC samples and 11 (41%) adjacent nontumor liver samples were HBxAg positive. No association with AFB_1–DNA adducts was observed for HBsAg and HBxAg. These methods should be useful in determining the role of exposure in the induction of HCC in Taiwan.

Introduction

Hepatocellular carcinoma (HCC) is one of the major cancers in the world (*1*) and the leading cancer for males in Taiwan, where hepatitis B virus (HBV) is hyperendemic (*2*). Hepatitis B virus has been established as one of the most important determinants of HCC; however, documented, independent risk factors of HCC include aflatoxin exposure, hepatitis C virus infection, alcohol drinking, cigarette smoking, and familial tendency (*3-6*).

To better determine exposure to aflatoxin at the individual level, monoclonal antibodies have been developed against the stable imidazole ring-opened form of the major guanine adduct of aflatoxin B_1 (AFB_1) (*7,8*). Antibodies recognizing this adduct were used in a competitive enzyme-linked immunosorbent assay (ELISA) to quantitate DNA adducts in liver DNA of animals treated with AFB_1 and in human liver tissue of HCC patients from Taiwan (*9*). In these initial studies on nine patients, adducts were detected in all tumor samples and in 25% of normal adjacent tissues. Adduct levels ranged from 1.2 to $3.5/10^6$ nucleotides.

Immunohistochemical Detection of Adducts in Rat Liver

For the development of immunohistochemcial methods for the detection of AFB_1–DNA adducts, rats were treated with several doses of AFB_1 ranging from 1 to 3.75 mg/kg and sacrificed after 2 hr (*10*). Frozen liver sections fixed in 70% ethanol were treated with 15 mM Na_2CO_3/30 mM $NaHCO_3$ (pH 9.6) for 2 hr at room temperature to ring open the guanine adducts. Liver sections were then treated with RNAse (100 μg/mL) at 37°C for 1 hr, with proteinase K (10 μg/mL) at room temperature for 10 min, and with 50 mM NaOH in 40% ethanol for 30 sec at room temperature to denature the DNA. Slides were then incubated with antibody 6A10 at 37°C for 45 min and with goat anti-mouse IgG conjugated with fluorescein isothiocyante (FITC) at 37°C for 45 min. They were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1 mg/mL) at 37°C for 45 min to allow

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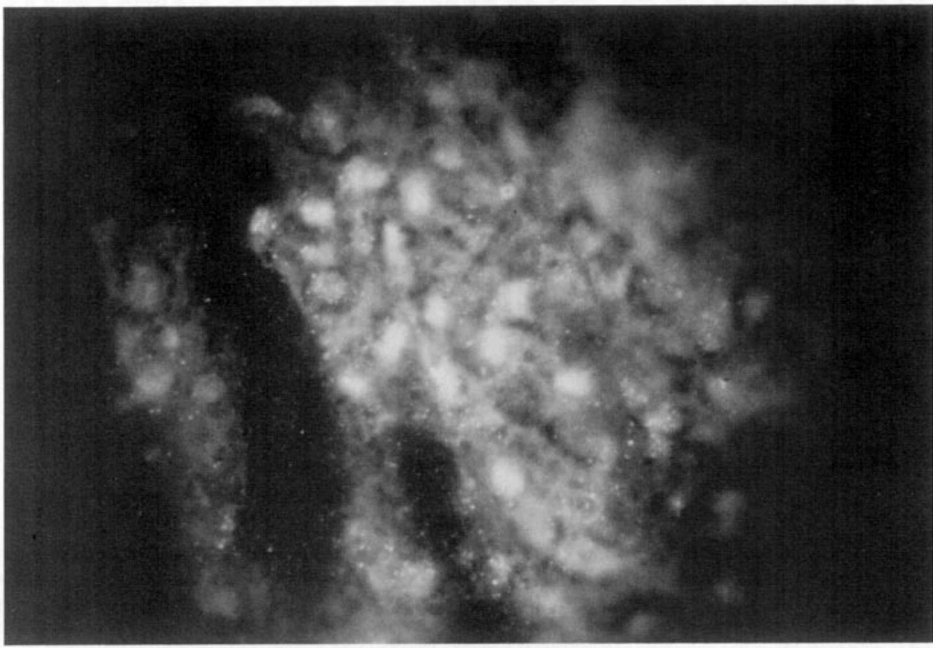
Combined FITC-DAPI staining was observed with a Nikon Optiphot microscope (Nikon, Tokyo). Photographs were taken with Kodachrome ASA400 slide film and fluorescence intensity of nuclei measured from the color slides using a Molecular Dynamics 300A densitometer (Molecular Dynamics, Sunnyvale, CA). The fluorescence intensity of each nucleus in a given liver section was determined by the difference in fluorescence intensity between the nucleus and its surrounding cytoplasm, as described *(10)*.

FITC staining was localized to the nuclei in animals treated with 2.5 mg/kg (Fig. 1). Sections from a control, untreated rat were negative, as were the additional controls of sections from a treated rat incubated with DNAse before staining or stained with antiserum that had been preabsorbed with AFB_1–DNA. Quantitative determination of relative fluorescence indicated a dose–reponse relationship between fluorescence intensity and dose of AFB_1. DNA was also isolated from liver tissue and adduct levels determined by competitive ELISA with antibody



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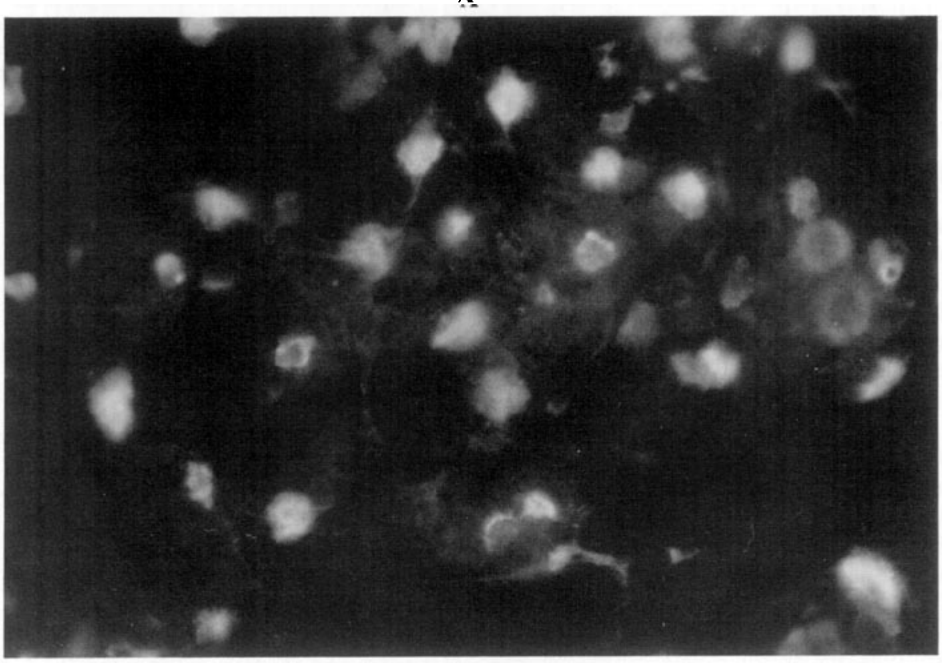

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FIGURE 1. Indirect immunofluorescence staining for imidazole ring-opened AFB_1–DNA adducts with antibody 6A10 and goat anti-mouse IgG-fluorescein isothiocyanate of *(A)* a liver section from a rat treated with 2.5 mg/kg AFB_1 and *(B)* a human liver section from Taiwan.

Table 1. Indirect immunofluorescence assay of AFB_1-DNA adducts and immunoperoxidase staining of HBsAg and HBxAg in HCC tumor and nontumor liver tissues from Taiwan.

Country of origin	Type of liver tissue	Indirect immunofluorescence				HBsAg/HBxAg								Total
		+		−		++		+/−		−/+		−/−		
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	Total
Taiwan	Nontumor	7	26	20	74	11	41	11	41			5	18	27
	Tumor	8	30	19	70	5	19	5	19	4	15	13	48	27
United States	Noncancer			5	100							5	100	5
	HCC			1	100	1	100							1
	Other tumors			2	100							2	100	2

Abbreviations: AFB_1, aflatoxin B_1; HBsAg, hepatitis B virus surface antigen; HBxAg, hepatitis B virus X antigen; HCC, hepatocellular carcinoma.

6A10 (*9*). There was a good correlation ($r = 0.61, p < 0.05$) between adduct levels determined by densitometric scanning of tissue sections and ELISA quantitation of an adducts on isolated DNA. In addition, an alternative method of analysis was used to confirm the immunological data. Quantitation of adduct levels by measurement of fluorescence spectra of liver DNA isolated from the treated animals correlated ($r = 0.78, p < 0.01$) with the densitometric scanning of immunohistochemically stained sections of liver tissue.

Immunohistochemical Detection of Adducts in Human Liver

The indirect immunofluorescence method was recently applied to the detection of AFB_1-DNA adducts in frozen liver tissue sections from Taiwan (*11*). A total of 27 pairs of surgically removed tumor and adjacent nontumor liver tissues from HCC patients were collected in the National Taiwan University Hospital. Figure *1B* shows a liver section positive for AFB_1-DNA adducts. Eight (30%) of the tumor and seven (26%) of the nontumor liver tissues of twenty-seven HCC patients from Taiwan had detectable levels of AFB_1-DNA adducts (Table 1). Eight liver samples from the United States, including one HCC obtained through the U.S. National Cancer Institute Cooperative Human Tissue Network, were also tested, but no adducts were detectable by indirect immunofluorescence.

Hepatitis B surface and X antigen (HBsAg and HBxAg) were detected with an avidin-biotin complex staining method (*11*). Both positive and negative staining for HBsAg and HBxAg were observed in the HCC tissues. For the 27 nontumor liver tissues, 41% were positive for both HBsAg and HBxAg, 41% for HBsAg alone, 0% for HBxAg alone, and 18% for neither antigen (Table 1). The corresponding frequencies were 19, 19, 15, and 48% for HCC tissues. The single U.S. HCC sample was positive for both HBsAg and HBxAg. All other U.S. samples were negative.

Table 2 gives the results of combining the status of HBV antigens and AFB_1-DNA adducts in paired HCC and adjacent nontumor liver tissues for each Taiwan patient. Although 24 (89%) of HCC patients had HBsAg and/or HBxAg in either HCC or adjacent nontumor liver tissues, 14 (52%) had detectable levels of AFB_1-DNA adducts in their HCC and/or adjacent nontumor liver tissues. There were 13 HCC patients who were positive for both HBV antigens and AFB_1-DNA adducts; 11 were positive for HBV antigens but not for adducts; 1 was positive for adducts only; and 2 were negative for both HBV antigens and adducts. There was no significant association between the carrier status of HBsAg and/or HBxAg and the detectable level of AFB_1-DNA adducts.

Table 2. Presence of hepatitis B virus antigens and AFB_1-DNA adducts in paired tumor and nontumor tissue of 27 HCC cases in Taiwan.

AFB_1-DNA adduct	Hepatitis B antigens					
	Positive		Negative		Total	
	No.	%	No.	%	No.	%
Positive	13	48	1	4	14	52
Negative	11	41	2	7	13	48
Total	24	89	3	11	27	100

Abbreviations: AFB_1, aflatoxin B_1; HCC, hepatocellular carcinoma.

Discussion

These studies demonstrate that quantitative immunohistochemical methods can be used to monitor exposure to aflatoxin B_1 by measurement of DNA adducts in liver tissue when present at levels higher than $1/10^6$ nucleotides. Samples obtained during surgery for HCC contained adducts at levels known to induce tumors in animals (*12*). Although these methods may not be useful for routine monitoring of healthy individuals, they are an important tool in understanding the role of AFB_1 in HCC. The results presented here suggest that AFB_1 may be involved in the development of HCC in Taiwan. Both adducts and viral antigens are present in the tumorous and nontumorous tissues in the liver of most HCC patients, but their relationship is not statistically significant. These results are compatible with the conclusion that HBV and AFB_1 do not act synergistically in the genesis of HCC, but further investigation will be necessary to define the relationship between HBV and AFB_1. We are currently implementing a case–control study of AFB_1-DNA to determine whether adduct levels are higher in HCC patients. This approach should provide more direct evidence for the role of aflatoxin in HCC.

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